Effect of Ewe Body Condition During Mid to Late Gestation on Progeny Mammary Epithelial Cell Proliferation

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Research Thesis

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ABSTRACT

Dam body condition score (BCS) during mid to late gestation may affect progeny mammary epithelial cell proliferation. To study this, pregnant ewes (n = 96; ≈80 d of gestation) were allotted to treatment groups based on initial BCS of 2, 3, or 4 (on a 1 to 5 scoring system with 1 being extremely thin and 5 being extremely fat). Ewes were housed in 18 pens (6 pens per treatment) and fed a diet of limit-fed corn silage (1.1 kg DMI/d), to which whole shelled corn was supplemented at 0.12, 0.26, and 0.47 kg DMI/d for BCS groups 2, 3, and 4, respectively. Diets were adjusted every 2 wk to maintain desired BCS throughout pregnancy. Prior to weaning, lambs nursed their mothers and were fed a common starter. Lambs were weaned (≈56 d of age; 23.59 kg) and placed on a common finishing diet that met NRC requirements. Female progeny from the three BCS groups (n = 73) were slaughtered at similar BW (46.9 ± 0.5 kg), and age (126.3 ± 2.8 d). Udders were removed and mid-parenchymal samples were obtained, fixed in formalin, and later embedded in paraffin blocks. Five-µm thick tissue sections were cut, mounted onto slides, and underwent immunohistochemical staining for the Ki67 antigen, a nuclear cell proliferation marker. Three digital pictures were captured per lamb and further analyzed to determine a Ki67 labeling index (number of Ki67 positive epithelial cells / number of total epithelial cells x 100). Data were analyzed with the Mixed Procedure of SAS using a model that included the effect of dam BCS and considered the covariates breed, parity, birth type, and rear type. Dam BCS tended to affect (P = 0.058) Ki67 labeling index of progeny. Numerically, female progeny of BCS 4 ewes had a lower Ki67 labeling index

(4.08 ± 0.72%), than either progeny of BCS 3 ewes (6.36 ± 0.79%), or progeny of BCS 2 ewes (6.27 ± 0.82%). These data suggest but do not demonstrate an effect of dam BCS during mid to late gestation on postnatal progeny mammary epithelial cell proliferation. Given that mammary epithelial cell number is positively correlated with milk yield, our observations here require further evaluation as they may have important lactation performance implications.

Key words: body condition score, sheep, mammary

INTRODUCTION

Nutrition has been shown to influence mammogenesis as early as the pre-weaning period in dairy heifers (Daniels et al., 2009) and the prepubertal period in ewe lambs (McCann et al., 2009). The effect of maternal nutrition on fetal mammary development in ewe lambs has recently been explored (van der Linden et al., 2009), but further research is needed in this area. Dam body condition score (**BCS**) during mid to late gestation may affect progeny postnatal mammary epithelial cell proliferation via in utero metabolic programming. This is a process whereby a stimulus or insult at a "sensitive" or "critical" period of development has lasting effects on the structure or function of the body (Barker, 2004). We hypothesized that mammary growth and development of ewe lambs might be affected by the gestational environment they experience.

Studies have shown that nutrition during fetal life affects fetal ovarian development (Borwick et al., 1997), postnatal growth (Greenwood et al., 1998), reproductive performance (Rae et al., 2002), and metabolism (Ravelli et al., 1998; Gardner et al., 2005; van der Linden et al., 2009). The objective of this study was to explore the effects of varying dam BCS during gestation on mammary epithelial cell proliferation in female progeny. The effects of maternal nutrition on the mammary gland may have implications on mammary growth so it is important to understand the mechanisms that control this. Although there are limited data to indicate that the extent of fetal mammary development is essential to subsequent milk production, the secretory epithelial cells that proliferate during pregnancy do so on the epithelial ducts that have developed during prenatal life (Jenkinson et al., 2003). The foundation for functional mammary secretory tissue, parenchyma (**PAR**), is established early in life; mammary epithelial cell number is positively correlated with

milk production. Milk is the sole source of nutrients for most newborn mammals; thus, the offspring's survival and potential to reach reproductive maturity are directly dependent upon the lactational success of its dam (van der Linden et al., 2009).

MATERIALS AND METHODS

Animals and Treatments

Animal procedures were approved by The Ohio State University's Institutional Animal Care and Use Committee. Lambs used were part of a larger trial studying the effects of ewe BCS during mid to late gestation on ewe performance and progeny postnatal growth (S.C. Loerch, OARDC, The Ohio State University, Wooster, OH, personal communication). Pregnant ewes (n = 96; ≈80 d of gestation) were allotted to treatment groups based on initial BCS of 2, 3, or 4 (on a 1 to 5 scoring system with 1 being extremely thin and 5 being extremely fat). Ewes were housed in 18 pens (6 pens per treatment) and fed a diet of limit-fed corn silage (1.1 kg DMI/d), to which whole shelled corn was supplemented at 0.12, 0.26, and 0.47 kg DMI/d for BCS groups 2, 3, and 4, respectively. Body weight and BCS were collected for two consecutive days at the start of the trial and every 2 wk during the trial. Diets were adjusted as needed to maintain similar BCS within treatments. Feed samples were taken every 2 wk, composited, and analyzed for dry matter, ether extract, neutral detergent fiber, acid detergent fiber, N, Ca, and P. Lamb weight and vigor score were recorded at parturition. Ewe weight and BCS were also recorded at that time.

Lactating ewes were housed in 18 pens (6 pens per treatment) and fed a common lactation diet. Lambs and ewes were weighed at 28 d postpartum (near peak lactation); milk production and composition were measured using a weigh-suckle-weigh approach.

Lambs nursed their own mother prior to weaning, which took place when offspring averaged 56.97 ± 0.99 d (P = 0.054) and 23.64 ± 0.92 kg (P = 0.525). Lambs then entered a feedlot phase, grouped by original dam pen, and were fed an identical finishing diet that met NRC requirements.

Mammary Gland Collection

Ewe lambs were harvested at an average age of 126.31 ± 2.80 d (P = 0.160) and body weight of 46.92 ± 0.53 kg (P = 0.913). Lambs were killed by use of captive bolt stunner followed immediately by exsanguination at The Ohio State University Meat Sciences Laboratory. The whole udder was removed, weighed, and bisected along the median suspensory ligament. Mid-PAR samples (~3mm x 3 mm x 3 mm) were obtained from the left mammary gland and fixed in a 10% formalin solution for 24 h. The formalin solution was then replaced with 70% ethanol and samples were stored at 4°C until they were processed for embedding. Mid-PAR samples were embedded in paraffin at The Ohio State University Department of Veterinary Biosciences.

Staining Procedures for Histology

Slides were processed similar to Daniels et al. (2009) and Brown et al. (2005). Briefly, microscope slides were prepared from mid-PAR samples by slicing 5-µm-thick sections from the paraffin-embedded tissue blocks with a microtome. Two or three serial tissue sections from each sample were mounted on positively charged microscope slides. Slides were deparaffinized in xylene (3 × 5 min), hydrated through a series of ethanol washes, quenched with 3% H_2O_2, and microwaved in a 10 mM citrate buffer (pH 6.0) for antigen retrieval. Before blocking, individual tissue sections were circled with a PAP barrier pen (cat no. 71312, Electron Microscopy Sciences, Hatfield, PA). Tissue sections

were then blocked with CAS block (cat no. 008120, Invitrogen, Carlsbad, CA) for 30 min before incubation with 200 µl of pre-diluted Ki67 rabbit monoclonal antibody (clone SP6, cat no. RM-9106-R7, Thermo Scientific, Waltham, MA) for 60 min. After rinsing slides in PBS to remove unbound primary antibody, slides were incubated for 30 min with 200ul of pre-diluted secondary antibody (broad spectrum poly-HRP conjugate; SuperPicture Polymer Detection Kit; Invitrogen, Carlsbad, CA). Tissue sections were then incubated with DAB chromogen (3,3' diaminobenzidine; Invitrogen, Carlsbad, CA) for 4 min. Slides were washed in deionized water and then counterstained with hematoxylin for 1 min. Slides were then washed in tap water, dehydrated, and then coverslipped with the aid of Permaslip mounting medium (Alban Scientific Inc., St. Louis, MO).

Image Acquisition and Analysis

Slides were viewed on an Olympus IX81 microscope (Olympus Corporation, Shinjuku-ku, Tokyo, Japan). Three digital images per slide (from the mid-PAR region of the udder for each animal) were captured with an Olympus DP72 camera. Images were selected at random by blurring the objective lens and moving the microscope stage prior to re-focusing. The microscope was set for brightfield illumination and 20× magnification. Lamp voltage was set at 9v, with natural contrast, and 0 exposure compensation. Each picture was white balanced, and the exposure length was locked at 83µs for all images. Images were processed using CellSens Standard Software (Olympus Corporation, Center Valley, PA) prior to final analysis. For this, images were individually opened in the CellSens program and processed using the "Count and Measure" tool. Ductal tissue was manually circumscribed with the computer mouse to create multiple regions of interest (**ROI**). After all ductal tissue within each picture was outlined as an ROI, a manual

threshold (or phase) was created to detect the Ki67 (brown) cells within the ROI (See **Figure 1** and **Figure 2**). The phase contained three channels with programmed minimum and maximum wavelengths of: Red, 109 and 530; Green, 37 and 426; and Blue, 30 and 461, respectively. Selection of the "Count and Measure on ROI" tool allowed only positive cells within ROI to be quantified. Ki67 is a nuclear antigen; to prevent non-nuclear artifacts from being counted, the object filter was set to exclude any objects smaller than $4\mu m^2$ (this area is smaller than the area of an average nucleus). The object filter was then removed and the remaining data were exported to a Microsoft Excel document. Within each image, each ROI was assigned a percentage of Ki67 positive cells by the software. These values were then summed and averaged for each image. The three image averages for each animal were then likewise summed and averaged, yielding a Ki67 labeling index (number of Ki67 positive cells / total number of epithelial cells × 100) for each animal.

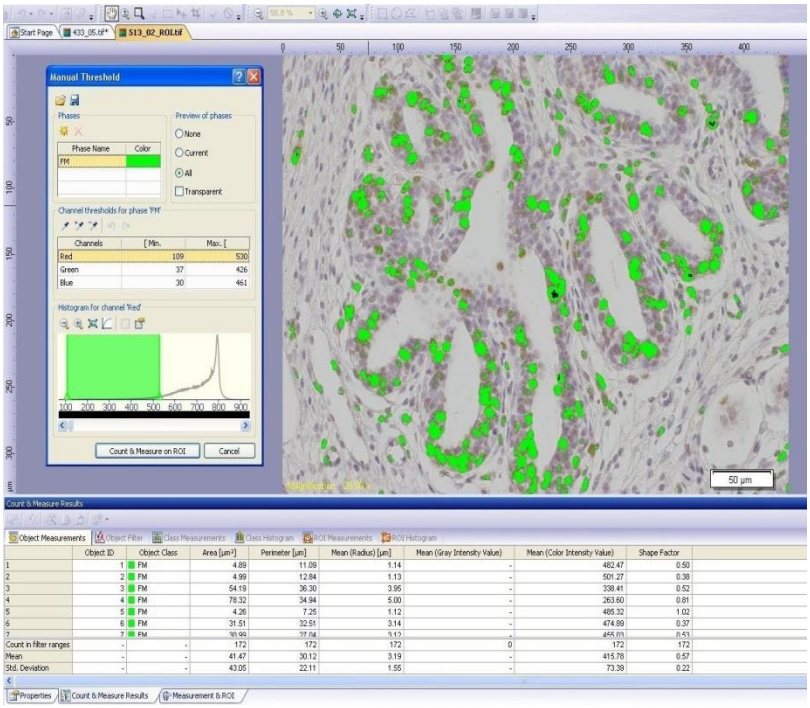

Figure 1. Prior to analyzing images, a manual threshold (phase) was created with CellSens Software to automatically detect all Ki67 positive cells (brown stain). This screen capture shows the created phase (green) highlighting all detected brown in the image.

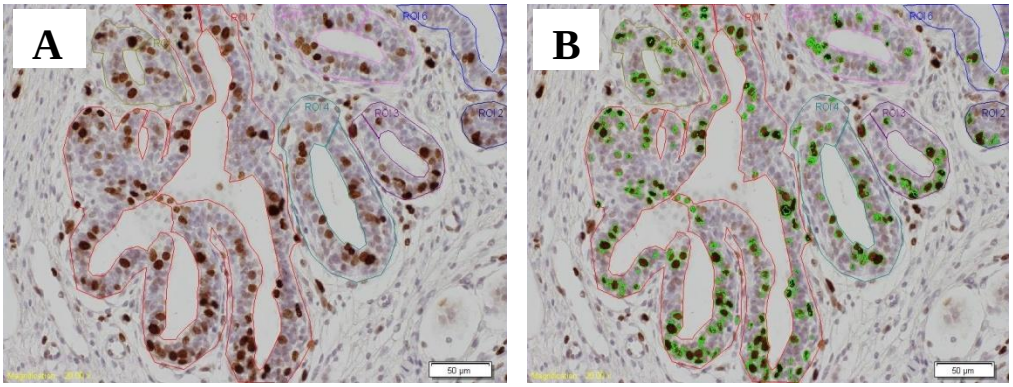

Figure 2. Image analysis. A) All ductal tissue was manually outlined by the user and classified by the software as a region of interest (**ROI**). B) The "Count and Measure on ROI" option was performed using the phase described in Figure 1. This ensured that only positive cells within ROIs were quantified. In other words, Ki67 positive cells in non-ductal areas were excluded from analysis.

Statistical Analysis

All statistical analyses were performed using the Mixed Procedure of SAS (version 9.2, SAS Institute, Cary, NC) with a model that included the effect of dam BCS. Breed, parity, birth type, and rear type were included in the model as covariates if they represented a significant ($P < 0.10$) source of variation. No random term was specified, yielding the basic model: $y_{ij} = \mu + T_i + e_{(i)j}$. "Lamb" was used as the experimental unit. When treatment was significant ($P \leq 0.05$), means were separated by the PDIFF procedure of SAS.

RESULTS

Dam BCS tended to affect ($P = 0.058$; **Figure 3**) progeny Ki67 labeling index (a measure of cell proliferation). Numerically, female progeny of BCS 4 ewes had a lower Ki67 labeling index (4.08 ± 0.72%), than either progeny of BCS 3 ewes (6.36 ± 0.79%), or progeny of BCS 2 ewes (6.27 ± 0.82%).

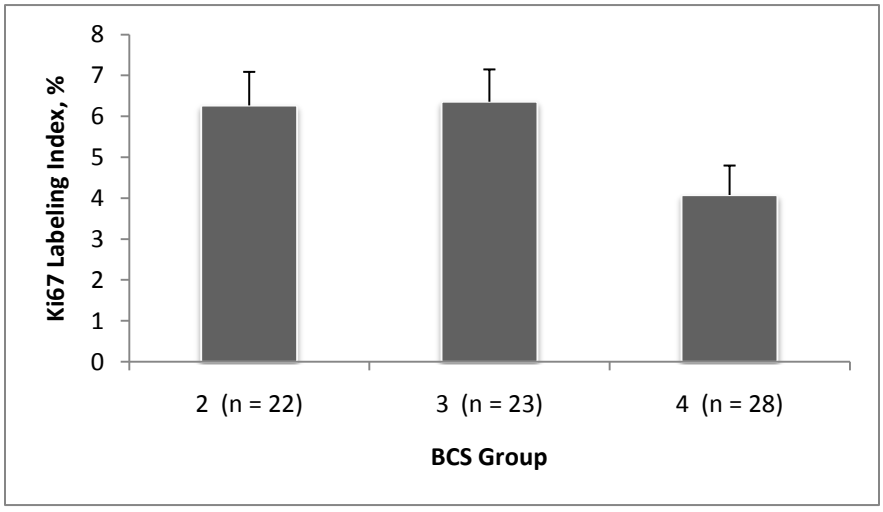

Figure 3. Ki67 labeling index (number of Ki67 positive cells / number of total epithelial cells x 100) data from female progeny of ewes maintained on one of three body condition score groups (**BCS**; 2, 3, or 4) during gestation. Data are LSmeans ± SEM.

DISCUSSION

The majority of ovine mammary growth occurs postnatally. Many factors applied postnatally, such as level of nutrient intake, are known to affect milk production of the dam. Scientists have known of these types of nutritional effects for many years in domestic animals (McCann et al., 1989).

The fetal period of mammary growth primarily consists of establishment of a primitive branching ductal network that differentiates postnatally into the alveolar system responsible for milk production. The effects of different fetal environments (nutrient intake of the mother) on mammary growth and subsequent first lactational performance of the *offspring* have not been extensively reported on; one of the only papers in this area is the work of van der Linden et al. (2009). Those authors suggest that ad-libitum feeding of the dam during pregnancy may have negative implications on lactation performance of the offspring (van der Linden et al., 2009). Although the current study focused on a single timepoint analysis of mammary epithelial cell proliferation in prepubertal ewe lamb offspring, our findings seemingly compliment the findings of van der Linden et al. (2009). Taken together, both studies suggest an inverse relationship between dam BCS during mid to late gestation and ewe lamb mammary epithelial cell proliferation and subsequent lactation milk yield. If this relationship really exists, then this demonstrates nutritional imprinting in the mammary gland.

As Capuco and Akers (2010) pointed out, appropriate nutrient intake and balance are important for mammary growth and development, and these processes may be

influenced by under-or over-nutrition. Our findings point to the importance of the fetal environment for mammary growth of the offspring.

CONCLUSION

In conclusion, dam BCS tended to affect Ki67 labeling index of progeny. We noted a potential inverse relationship between dam BCS during mid to late gestation and ewe lamb mammary epithelial cell proliferation. Given that mammary epithelial cell number is positively correlated with milk yield, our observations here require further evaluation as they may have important lactation performance implications for sheep as well as dairy cattle, by extension.

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